



Small molecule probes for plant cell wall polysaccharide imaging

Ian S. Wallace^{1,2} and Charles T. Anderson^{3*}

¹ Energy Biosciences Institute, University of California, Berkeley, CA, USA

² Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA

³ Department of Biology, The Pennsylvania State University, University Park, PA, USA

Edited by:

Seth DeBolt, University of Kentucky, USA

Reviewed by:

Stephan Pollmann, Universidad

Politécnica de Madrid, Spain

Kian Hématy, Institut National de la

Recherche Agronomique, France

*Correspondence:

Charles T. Anderson, Department of Biology, The Pennsylvania State University, 201 Life Sciences Building, University Park, PA 16802, USA.

e-mail: cta3@psu.edu

Plant cell walls are composed of interlinked polymer networks consisting of cellulose, hemicelluloses, pectins, proteins, and lignin. The ordered deposition of these components is a dynamic process that critically affects the development and differentiation of plant cells. However, our understanding of cell wall synthesis and remodeling, as well as the diverse cell wall architectures that result from these processes, has been limited by a lack of suitable chemical probes that are compatible with live-cell imaging. In this review, we summarize the currently available molecular toolbox of probes for cell wall polysaccharide imaging in plants, with particular emphasis on recent advances in small molecule-based fluorescent probes. We also discuss the potential for further development of small molecule probes for the analysis of cell wall architecture and dynamics.

Keywords: plant cell wall, cell wall dynamics, cellulose, pectin, click chemistry

INTRODUCTION

Terrestrial plants annually assimilate 1.3×10^{11} metric tons of CO₂ through the process of photosynthesis (Beer et al., 2010), and the majority of the resulting photosynthate is used to produce the polysaccharide-rich walls that surround plant cells (Pauly and Keegstra, 2008). These complex extracellular matrices are composed of cellulose microfibrils (CMFs), neutral hemicelluloses, acidic pectins, and proteins and must simultaneously be strong enough to resist the substantial cellular osmotic pressure necessary to support turgor-mediated cell growth and dynamic enough to allow cell expansion to occur (Somerville et al., 2004; Cosgrove, 2005). As a result, the ordered deposition of plant cell walls determines the patterning and extent of plant growth and development at the cellular level (Desnos et al., 1996; Arioli et al., 1998; Nicol et al., 1998).

Despite the physiological importance of cell wall deposition and dynamics, relatively little is known about cell wall structure in living cells, primarily due to a paucity of effective labeling strategies for polysaccharides in their native environment. Genetically encoded fluorescent tags for polysaccharides do not exist, necessitating novel approaches for the specific labeling of cell wall glycans. Although chemical (Reiter et al., 1997; Brown et al., 2007) and biochemical (Lerouxel et al., 2002; Barton et al., 2006; Bauer et al., 2006) techniques can accurately determine the primary structures of polysaccharides, these methods provide few spatial details due to the necessity for sample homogenization. Imaging approaches, such as transmission electron microscopy (Moore et al., 1991; Lynch and Staehelin, 1992), Raman microspectroscopy (Schmidt et al., 2010), and fluorescence microscopy with an array of polysaccharide-specific antibodies, lectins, and carbohydrate binding domains (Pattathil et al., 2010) have provided qualitative information regarding the localization and relative abundance of numerous cell wall polysaccharides. However, these techniques

are limited by long sample preparation times, potential fixation artifacts, loss of temporal information, lack of epitope specificity (Pattathil et al., 2010), and complications in interpretation due to epitope masking (Marcus et al., 2008). In addition, protein-based probes, particularly intact 160 kDa IgG molecules, are very large compared to the approximately 3 nm pore size of cell walls (Chesson et al., 1997) and may not efficiently label some cell wall components (Marcus et al., 2010). As a result, many developmental changes in cell wall architecture cannot be fully characterized using these approaches.

Polysaccharide-specific fluorescent probes and metabolic labels augment the currently available cell wall imaging toolbox, with the added benefit that these probes are compatible with live-cell imaging and/or pulse-chase experiments. In this review, we summarize the recent development of specific small molecule chemical probes for cell wall imaging, and the insights that these new probes have provided concerning plant cell wall structure.

CELL WALL POLYSACCHARIDE BIOSYNTHESIS

The structural complexity of the plant cell wall arises in part due to the variety of polysaccharides used to assemble this organelle, the spatial and temporal separation of their synthesis, and the various chemical modifications that occur within these polysaccharides. Cell wall polysaccharides are often grouped into three functional categories: cellulose, hemicelluloses, and pectins. Cellulose is composed of 30–40 β-(1 → 4)-linked glucan chains that associate to produce semi-crystalline CMFs (Somerville, 2006). These structural polysaccharides are synthesized at the plasma membrane by 30 nm “rosette” complexes (Arioli et al., 1998; Kimura et al., 1999) containing multiple cellulose synthase A (CesA) catalytic subunits (Taylor et al., 2003; Desprez et al., 2007; Persson et al., 2007). Live-cell imaging has indicated that CesA-containing complexes co-align with cortical microtubules underlying the plasma

membrane as they produce nascent CMFs (Paredes et al., 2006; Li et al., 2012), which are deposited in the apoplast.

In contrast to CMFs, hemicelluloses and pectins are synthesized in the Golgi (Mohnen, 2008; Caffall and Mohnen, 2009) and delivered to the apoplast by vesicle-mediated exocytosis via an incompletely described pathway (Moore et al., 1991; Lynch and Staehelin, 1992). Once deposited in the apoplast, hemicelluloses, such as xyloglucan, are thought to associate with and crosslink CMFs in the cell wall (Vincken et al., 1995; Lima et al., 2004), forming a cellulose–hemicellulose network. The interaction between these polysaccharides is proposed to prevent individual CMFs from interacting with each other and to help organize CMFs into a cohesive, dynamic network, although this model has recently been challenged (Park and Cosgrove, 2012b). Hemicelluloses and CMFs are embedded in a gel-like matrix composed of the pectic polysaccharides homogalacturonan, xylogalacturonan (XGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) which together control aspects of cell adhesion, wall extensibility, and wall porosity (Krupkova et al., 2007; Mohnen, 2008).

Plant cell wall polysaccharides can also be modified during synthesis or after deposition in the apoplast by the activity of various enzymes. Pectins can be reversibly acetylated at the O-2 or O-3 hydroxyl groups of their galacturonic acid backbone sugars and/or methylesterified at the O-6 carboxyl group (Ishii, 1997; Manabe et al., 2011). Hemicelluloses can also be acetylated (Pauly et al., 1999; Kabel et al., 2003; Gibeaut et al., 2005; Gille et al., 2011) or have their glycan side chains modified during plant development (Günl et al., 2011). Finally, transglycosylase enzymes, such as xyloglucan endotransglycosylase (XET), cut and religate individual xyloglucan chains during wall extension (Xu et al., 1995; Maris et al., 2009). These modifications can significantly affect the physical properties of individual wall polymers (Huang et al., 2002; Ngoumazong et al., 2012) and of the cell wall as a whole (Krupkova et al., 2007; Peaucelle et al., 2008).

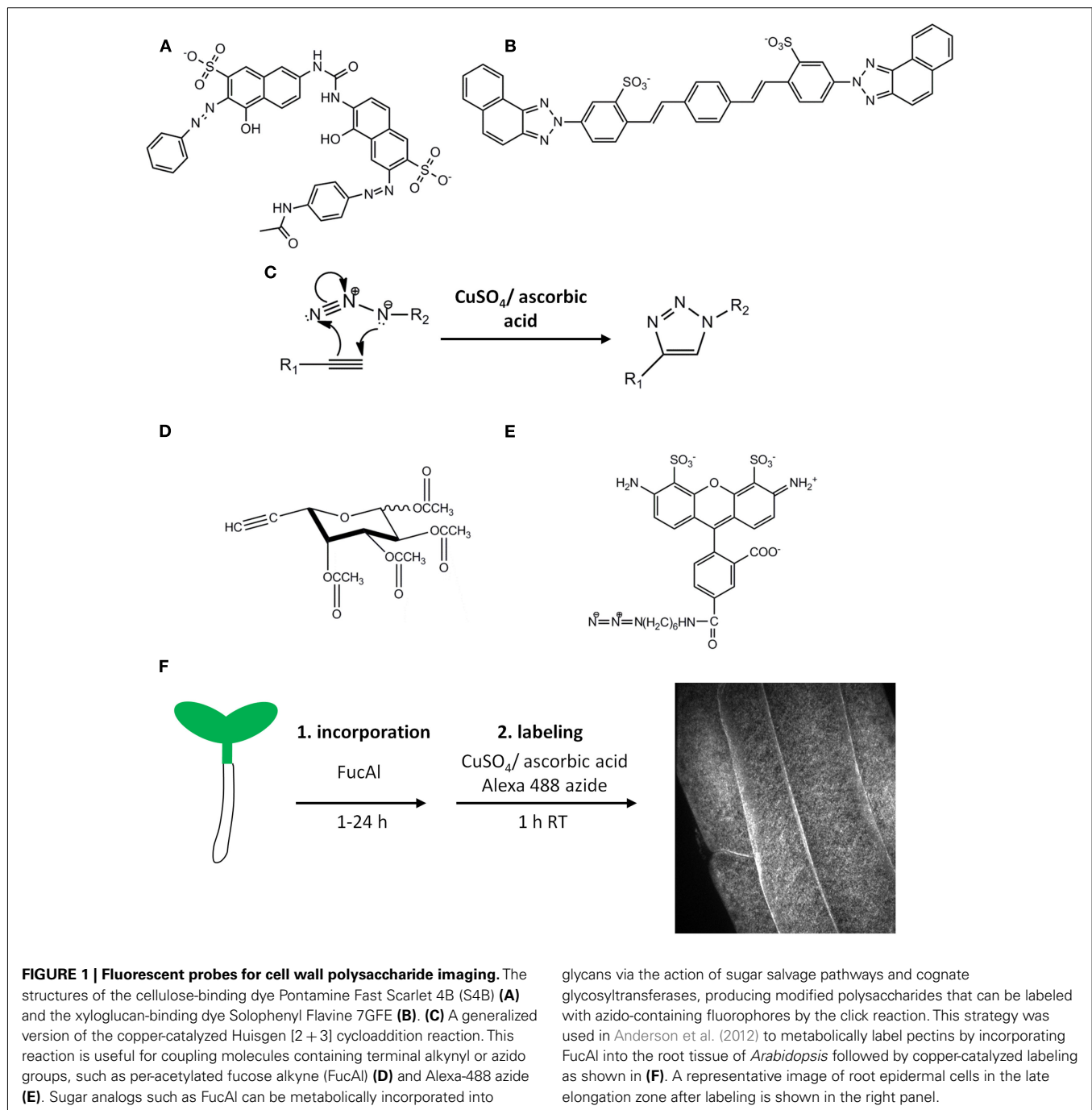
POLYSACCHARIDE-BINDING DYES AND METABOLIC LABELS FOR CELL WALL POLYSACCHARIDE IMAGING

Cell wall polysaccharides are structurally diverse, exist in complex three-dimensional arrangements, and are synthesized, deposited, modified, and degraded in highly dynamic processes. Therefore, probes that are highly specific for an individual polysaccharide type and compatible with live-cell imaging can effectively capture this complexity. Various polysaccharide-binding dyes, including Calcofluor white (Fagard et al., 2000; Homann et al., 2007; Harpaz-Saad et al., 2011), Congo red (Kerstens and Verbelen, 2002), ruthenium red (Western et al., 2001; Marks et al., 2008; Harpaz-Saad et al., 2011), and Aniline blue (Adam and Somerville, 1996; Nishikawa et al., 2005; Nguyen et al., 2010; Xie et al., 2011), have been used as imaging tools for plant cell walls, but these dyes cross-react with multiple polysaccharides, and their direct glycan targets are often unknown. To address this issue, Anderson et al. (2010) assayed the previously described fungal cell wall-binding dyes Pontamine Fast Scarlet S4B (S4B) and Solophenyl Flavine 7GFE (7GFE; Hoch et al., 2005) for their ability to bind isolated cell wall polysaccharides. S4B (Figure 1A) exhibits several useful imaging properties, including increased fluorescence

intensity in the presence of cellulose, increased glycan specificity over Calcofluor white, and spectral properties compatible with 532 and 561 nm lasers. S4B is also non-toxic at concentrations that are compatible with fluorescent imaging and can thus be used as a specific probe to dynamically image cellulose in living plant tissues. 7GFE (Figure 1B) exhibits a characteristic shift in its fluorescence emission maximum in the presence of xyloglucan, but not cellulose. Whereas 7GFE is slightly more toxic than S4B, this dye could potentially serve as an orthogonal fluorescent label for xyloglucan because its excitation and emission maxima do not overlap with those of S4B (Anderson et al., 2010).

S4B, 7GFE, and other fluorescent polysaccharide-binding probes are valuable tools that should be explored further, but the biophysical details of many dye–polysaccharide interactions are currently unknown, preventing the rational design of new dyes. However, alternative labeling strategies can be employed to design metabolic probes that can be targeted to individual cell wall polysaccharide networks. For example, fluorescently labeled xyloglucan fragments are metabolically incorporated into subdomains of xyloglucan by XETs, and these oligosaccharides have been used to image the sites of XET activity during cell expansion (Vissenberg et al., 2000). Additionally, molecules containing terminal alkynyl or azido functional groups can participate in a copper-catalyzed [2 + 3] cycloaddition referred to as the Huisgen “click” reaction (Figure 1C), which is bio-orthogonal, proceeds rapidly at room temperature with high specificity, and results in the formation of a stable triazole ring (Kolb et al., 2001; Rostovtsev et al., 2002; Tornøe et al., 2002). Exploiting this reaction, small, biologically relevant molecules containing these functional groups have been synthesized and incorporated into proteins, fatty acids, nucleic acids, and sugars which are then covalently labeled with useful probes, such as fluorophores, epitope tags, or other affinity reagents that contain the opposite functional group (Chin et al., 2002; Kiick et al., 2002; Deiters et al., 2003; Kho et al., 2004; Link et al., 2006; Rabuka et al., 2006; Hang et al., 2007; Hsu et al., 2007; Jao and Salic, 2008; Salic and Mitchison, 2008; Chang et al., 2009; Kaschani et al., 2009). These chemical methods have been particularly useful for imaging glycan conjugates in animal, bacterial, and fungal systems (Prescher et al., 2004; Dube et al., 2006; Laughlin et al., 2008), but had not previously been explored in the context of the plant cell wall.

Anderson et al. (2012) investigated the potential of click chemistry to label cell wall polysaccharides using per-acetylated fucose alkyne (FucAl; Figure 1D) as a suitable click sugar monosaccharide analog. FucAl was metabolically incorporated into *Arabidopsis* root tissue and labeled with Alexa-488 azide using the copper-catalyzed click reaction (Figures 1D–F). Cytological and biochemical analyses indicated that the majority of the FucAl label was incorporated into the pectic fraction of *Arabidopsis* cell walls, most likely in arabinogalactan side chains of rhamnogalacturonan-I (RG-I). Fucose is present in multiple cell wall polysaccharides (Nakamura et al., 2001; Lerouxel et al., 2002; Glushka et al., 2003; Strasser et al., 2004; Wu et al., 2010), but *Arabidopsis* mutants lacking fucosyltransferases for xyloglucan (Vanzin et al., 2002), arabinogalactan protein (Wu et al., 2010), and N-linked glycoproteins



(Strasser et al., 2004) incorporated FucAl at levels similar to wild-type plants, suggesting that FucAl does not evenly label all fucosylated cell wall glycans (Anderson et al., 2012). We hypothesize that this selectivity might arise from conformational differences in the active sites of different fucosyltransferases, suggesting that click chemistry-compatible sugar analogs might be selectively incorporated into specific polysaccharide networks even if the parent monosaccharide is present in several polymers. Structure–activity relationships of click sugar variants containing the alkyne or azide at different positions may elucidate the structural requirements for selective monosaccharide analog incorporation.

POLYSACCHARIDE IMAGING PROBES HIGHLIGHT THE STRUCTURAL DYNAMICS OF CELL WALLS DURING DEVELOPMENT

S4B staining and spinning disk confocal microscopy were used to image CMFs in *Arabidopsis* root tissues (Anderson et al., 2010), which represent a developmental continuum of dividing, expanding, and differentiated cells (Dolan et al., 1993). S4B-stained CMFs were transversely oriented with respect to the major cellular expansion axis in recently divided cells near the root cap and cells in the early elongation zone. However, older cells in the elongation zone exhibited diagonally oriented CMFs, and fully differentiated cells

that were no longer expanding contained longitudinally oriented CMFs (**Figure 2A**). This developmental gradient of CMF orientation agrees with previous electron microscopy studies describing developmental changes in CMF orientation along *Arabidopsis* roots and hypocotyls (Sugimoto et al., 2000; Refregier et al., 2004), suggesting that S4B staining provides a rapid and specific method for CMF imaging.

FucAl incorporation and labeling has been used to image pectic networks along the same root developmental continuum. After 1 h of incorporation, FucAl labeling in the root elongation zone appeared as small puncta in the cell wall (Anderson et al., 2012), potentially representing the locations of vesicle-mediated pectin delivery to the apoplast. After longer incorporation times (e.g., 12 h), the pattern of FucAl labeling in *Arabidopsis* roots exhibited a developmental gradient from the early elongation zone to the differentiation zone. In recently divided cells, FucAl-associated fluorescence was homogeneous, likely as a result of cumulative evenly distributed vesicle-mediated polysaccharide delivery. However, cells in the late elongation zone exhibit more organized patterns of puncta that transition to diagonally oriented fibrillar structures in the differentiation zone (Anderson et al., 2012). These observations suggest that pectic wall material is delivered and organized in distinct patterns during cellular development and that this developmental gradient parallels the reorientation of CMFs during cell elongation (**Figure 2B**).

Polysaccharide-specific probes can also serve as useful tools for the detailed characterization of mutants affecting cell wall polymer networks. For example, S4B staining has revealed new aspects of wall morphology in previously described *Arabidopsis* mutants defective in cellulose and xyloglucan biosynthesis. The *CesA6* mutant *Procuste1-1* (*prc1-1*; Desnos et al., 1996) produces less crystalline cellulose in its cell walls (Fagard et al., 2000), and *prc1-1* seedlings exhibited reduced S4B staining compared to wild-type seedlings (Anderson et al., 2010). Additionally, microfibrils in the root elongation zone were more defined but less prevalent, suggesting that fewer intact microfibrils were synthesized, leading to compromised cell walls and increased isotropic expansion of root cells. S4B staining of the *xtt1*; *xtt2* mutant, which lacks detectable amounts of xyloglucan (Cavalier et al., 2008; Park and Cosgrove, 2012a), labeled CMF fibers that were more prominently defined and thicker than wild-type CMF fibers, supporting the hypothesis that hemicelluloses prevent CMFs from interacting with each other in wild-type primary cell walls. Furthermore, S4B was recently used to characterize cellulose architecture in *Arabidopsis* seed coat mucilage (Harpaz-Saad et al., 2011), leading to the identification of *Cellulose Synthase A5* (*CesA5*), *FEI2*, and *Salt Overly Sensitive 5* (*SOS5*) as genes responsible for mucilage cellulose deposition. This study indicates that fluorescent probes like S4B will be useful for cell wall imaging and mutant phenotype identification in multiple developmental contexts.

DYNAMIC REORIENTATION OF CELL WALL POLYSACCHARIDE NETWORKS

Due to the load-bearing function of CMFs in the cell wall, differences in the orientation of these polymers with respect to the growth axis of anisotropically expanding cells has been proposed to critically affect cell shape (Green, 1960). When CMFs are deposited transversely, they resist radial expansion but allow cells

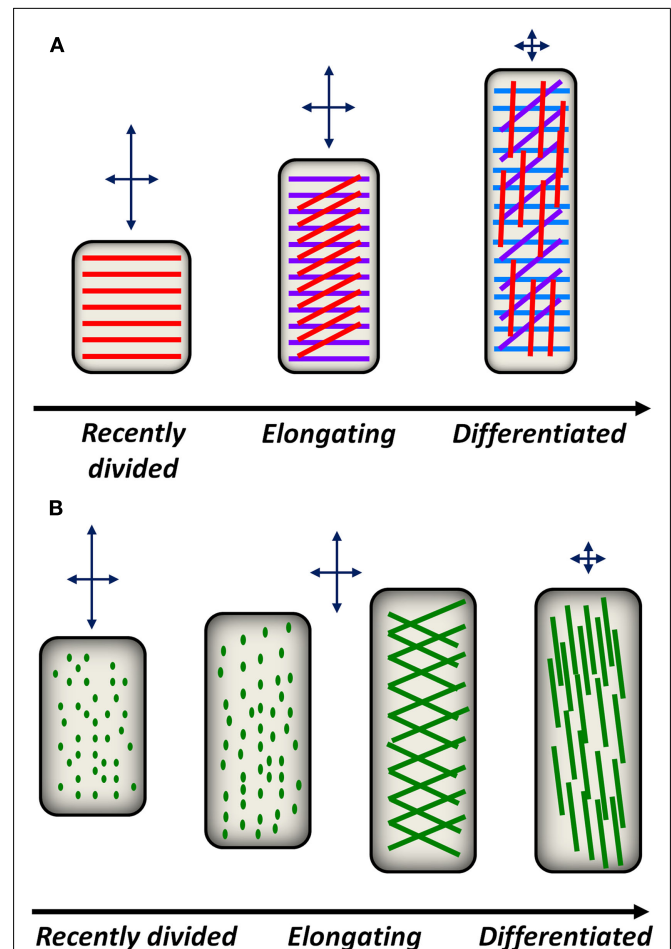


FIGURE 2 | Conceptual models for cell wall polysaccharide deposition and reorientation during plant development. (A) A model for cellulose deposition and reorientation in anisotropically elongating root epidermal cells. Shortly after division in the root meristematic region, cellulose synthase complexes in epidermal cells deposit cellulose (red lines) in a transverse orientation with respect to the elongation axis. This deposition pattern constrains radial expansion of the cell but permits axial expansion as indicated by the arrows above each cell. As epidermal cells elongate, new cellulose layers (magenta lines, blue lines) are deposited transversely, while older layers in the apoplast passively reorient from transverse to diagonal, then longitudinal orientations. The reorientation of older cellulose layers reduces the ability of differentiating cells to expand in the axial direction. **(B)** A model for pectin deposition and remodeling in the apoplast. In recently divided cells, pectic polysaccharides are delivered to the apoplast by secretory vesicles. Putative sites of pectin delivery (green dots) can be imaged with FucAl and are initially distributed evenly across the cell. As epidermal cells stop elongating, diagonal fibrils begin to appear that eventually reorient toward the longitudinal axis, potentially constraining further axial expansion.

to expand axially. However, as CMFs become longitudinally oriented and parallel to the elongation axis, cells are also expected to encounter expansion resistance in the axial direction (**Figure 2A**). Previous observations suggest that varying CMF angles may result from reorientation of these polysaccharides after deposition in the apoplast (Refregier et al., 2004). Optical sectioning of S4B-stained tissue indicates that CMFs in the outer cell wall layers of differentiated root epidermal cells are oriented longitudinally with respect

to the cellular growth axis, whereas CMFs near the inner face of the wall are oriented transversely (Anderson et al., 2010). Time-lapse imaging of S4B-stained CMFs revealed that the observed changes in orientation are attributable to the rotation of CMFs after they are deposited in the apoplast of root epidermal cells.

FucAl incorporation and labeling was also used to examine the dynamics of pectic networks in *Arabidopsis* (Anderson et al., 2012). Whereas the amounts of FucAl required for incorporation experiments had no effect on normal *Arabidopsis* seedling development, the Cu (I) necessary to catalyze the cycloaddition reaction was toxic, in agreement with previous *in vitro* and *in vivo* observations in other organisms (Agard et al., 2004; Brewer, 2010; Lallana et al., 2011). As an alternative to *in vivo* imaging, plants were pulsed with FucAl for 1 h and labeled at various chase times (Anderson et al., 2012). These experiments revealed that FucAl-associated fluorescence was initially punctate in the apoplast, but appeared as longitudinal fibers after 12 or 24 h chase periods, suggesting that, like CMFs, pectins become longitudinally oriented over time. As a whole, these observations suggest that polysaccharide networks are highly dynamic during cellular development and highlight the importance of specific polysaccharide-directed probes that are capable of capturing these events.

CONCLUSION AND FUTURE DIRECTIONS

The use of multiple labeling strategies for plant cell wall polysaccharides is altering the perception of the cell wall from that of a rigid case to that of a highly dynamic extracellular organelle that changes during development. However, the complexity of the cell wall necessitates the design of specific imaging probes for these dynamic events. Polysaccharide-binding dyes, such as S4B and 7GFE, highlight the utility of fluorescent dyes that are compatible with live-cell imaging and exemplify one avenue for the implementation of new cell wall imaging tools for the future. Indeed, we believe that novel cell wall imaging probes will most likely be isolated via high-throughput fluorescent interaction assays against isolated wall components, structure–activity studies of existing fluorophores, or array-based selectivity analyses that have been described for polysaccharide-binding antibodies (Moller et al., 2008; Pattathil et al., 2010; Sorensen and Willats, 2011). These experiments may lead to the identification of novel fluorophores that label unique polysaccharide populations or

networks, so that multiple cell wall polysaccharides can be imaged simultaneously.

Monosaccharide analogs compatible with click chemistry have some advantages over polysaccharide-specific fluorophores that suggest their utility in a variety of experiments. Because click sugars are incorporated metabolically, they label only those polysaccharides synthesized during the incorporation period and are therefore distinct from probes that label all of the existing polysaccharides of a given type. These sugars have the potential to serve as valuable tools for examining the trafficking of polysaccharides from the Golgi to the apoplast, elucidating extracellular events that occur after deposition of polysaccharides in the wall, and the isolation of mutants that affect these processes. These experimental goals will be furthered by the application of click sugars that label other polysaccharide networks, and it may be possible to tune the selective incorporation of a given sugar analog by changing the position or identity of the alkynyl or azido functional group. Membrane permeable click-compatible coumarin and naphthalamide fluorescent probes have been described (Sawa et al., 2006; Hsu et al., 2007), and could be used to image intracellular sites of polysaccharide synthesis to complement surface-specific labeling by membrane impermeable fluorophores, such as Alexa Fluor dyes. Additionally, *in vivo* click labeling using either copper-free strain-promoted [2 + 3] cycloaddition reactions (Prescher et al., 2004; Dube et al., 2006; Laughlin et al., 2008) or chelators that sequester toxic Cu (I) (Chan et al., 2004; Lewis et al., 2004) might be applicable to plant systems and could further facilitate the imaging of cell wall glycans in living cells. By adding a temporal dimension to studies of cell wall architecture, the approaches described here provide new ways of examining the synthesis, deposition, and dynamics of plant cell walls that will further our understanding of these remarkable bio-materials, both in the context of plant development and as renewable resources for human use.

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